Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 4219–4222, June 1986
Biochemistry

Both ATP and the electrochemical potential are required for optimal assembly of pro-OmpA into *Escherichia coli* inner membrane vesicles

(secretion/F_1F_0-ATPase/bacterial export energetics)

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Communicated by Paul D. Boyer, February 21, 1986

ABSTRACT **Pro-OmpA is processed to OmpA by isolated inverted plasma membrane vesicles from *Escherichia coli*. In the presence of ATP and a membrane potential, 58% (±13%) of the OmpA is sequestered in the vesicles. We sought to determine which of these two metabolic energy sources is used for protein translocation. The plasma membrane F_1F_0-ATPase is the central enzyme that interconverts the energy of membrane electrochemical potential and ATP. To separate the effects of these two forms of energy *in vitro*, the ATPase was inactivated, either by "stripping" the F_1 from the membranes with low salt and EDTA or by using membrane vesicles derived from a strain without the *atp* operon. In each case, optimal translocation and processing of pro-OmpA required both a membrane potential and ATP. We conclude that ATP and membrane potential are separate requirements for bacterial protein export.**

Protein uptake into mitochondria and chloroplasts, as well as bacterial protein export, requires metabolic energy either in the form of high-energy phosphate (as ATP) or transmembrane electrochemical potential. Chloroplast protein import has been reported to require ATP (1), while mitochondrial protein import requires the electrochemical potential across the inner mitochondrial membrane (2–4). *In vivo* studies showed that the export of M13 procoat across the inner membrane of *Escherichia coli* requires the electrochemical potential (5). This has been found by workers in several laboratories to be a general requirement for protein export in this organism (6–9).

Recently, two groups (10–14) have reported similar cell-free translocation reactions. These require ATP for the transfer of bacterial pre-proteins synthesized *in vitro* into sealed inverted plasma membrane vesicles. Müller and Blobel (14) reported that the F_1-ATPase is essential for this *in vitro* protein translocation, suggesting that the ATP is needed primarily to generate an electrochemical potential, which drives the assembly event. Chen and Tai (11) have shown that ATP is required directly for transport, and they have suggested that the electrochemical potential has, at most, a small effect. This suggestion is in conflict with the *in vivo* studies cited above as well as the *in vitro* studies of Müller and Blobel (14). However, the two groups used different *in vitro* reaction conditions and different techniques to test the energy requirement. Moreover, in these studies, the electrochemical potential under the various conditions of assay was not reported by either group.

Separation of the effects of ATP and membrane potential is complicated by the fact that the plasma membrane F_1F_0-ATPase generates a membrane potential at the expense of ATP. We have therefore prepared membrane vesicles with inactivated ATPase and, in the presence of ATP and/or NADH (which can generate a potential through NADH oxidase), measured both the membrane potential and the uptake of pro-OmpA. We find that both ATP and the membrane potential are essential for optimal protein translocation.

MATERIALS AND METHODS

Materials. Ampicillin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), chloramphenicol, chymostatin, chymotrypsin, ovoinhibitor, phenylmethylsulfonyl fluoride, putrescine, and spermidine were from Sigma. RNA polymerase was from Boehringer Mannheim, and Sephadex was from Pharmacia. 9-Amino-6-chloro-2-methoxyacridine was a gift from Robert D. Simoni (Stanford University).

Bacterial Strains. The following *E. coli* K-12 strains were used: MC1061 (F^-; *araD 139Δ(ara-leu)7697Δlac galU galK hsr hsm⁺ rpsL*), D10 (*rna-10, relA1, spoT1, metB1*), and DK8 (*bglR, thi-1, rel-1, HfrPO1,Δ(uncB-uncC) ilv*::Tn*10*). DK8 was kindly provided by Robert D. Simoni. D10 was grown in Luria broth and DK8 was grown in Luria broth plus tetracycline (15 μg/ml).

Membrane Preparation. Inverted inner membranes were prepared as described by Rhoads *et al.* (10).

Cell-Free Protein Synthesis. ^{35}S-labeled pro-OmpA was synthesized in a cell-free reaction as described (15), except that the reaction included carrier-free [^{35}S]methionine (1.4 mCi/ml; 1 Ci = 37 GBq), 29 mM NaOAc, RNA polymerase (36 units/ml), and plasmid DNA (121 μg/ml) (see below). Synthesis was stopped by addition of chloramphenicol and methionine to final concentrations of 250 μg/ml and 20 mM, respectively. The reaction was cooled on ice and passed through a column of Sephadex G-25 equilibrated in 50 mM Hepes/1 mM dithiothreitol, pH 7.5. The void volume fractions, which contained the ^{35}S-labeled pro-OmpA, were pooled. Chloramphenicol was added (250 μg/ml), and the pooled material was immediately used in the translocation reaction.

Translocation Reaction. The translocation reaction contained 1 mM spermidine, 8 mM putrescine, 1 mM dithiothreitol, 50 mM Hepes (pH 7.5), 40 mM KCl, 5 mM $MgCl_2$, and 50 μl of ^{35}S-labeled pro-OmpA in a final vol of 70 μl. Where indicated in the figure legends, the mixture also contained 10–22 μg of inner membranes, 1 mM ATP, and 2 mM NADH. Components were mixed at 0°C, incubated at 40°C for 17 min, and then cooled on ice. A portion was prepared for electrophoresis; the remainder was divided into two aliquots, and each was mixed with chymotrypsin (110 μg/ml). One of the

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Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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aliquots was mixed with 2% octyl-β-D-glucopyranoside, and both were incubated on ice for 1 hr. Ovoinhibitor (2 mg/ml), phenylmethylsulfonyl fluoride (0.25 mM), and 1/3rd vol of an aqueous saturated solution of chymostatin were added, and the incubation was continued on ice for 30 min. The samples were immunoprecipitated (9) and analyzed by $NaDodSO_4$/PAGE and fluorography (16).

Plasmid DNA Purification. *E. coli* MC1061 was transformed with a pBR322 derivative containing the *OmpA* gene under the λ phage promoter P_L. This transformant also bears a second compatible plasmid, pCI857, which codes for a temperature-sensitive λ repressor. Cells were grown in LB medium plus ampicillin (100 μg/ml) at 30°C to midlogarithmic phase. Chloramphenicol was added (170 μg/ml), and the incubation was continued for 15 hr. The cells were harvested and the plasmids were purified as described by Davis *et al.* (17).

Removal of F_1-ATPase from the Membrane Vesicles. The F_1-ATPase was stripped from the membrane vesicles as described (18). Briefly, D10 membrane vesicles (0.33 ml, 1 mg/ml) were incubated for 2 hr on ice in 1 mM Tris·HCl/1 mM EDTA, pH 7.5/10% (vol/vol) glycerol/2.5 mM 2-mercaptoethanol. The vesicles were centrifuged (150,000 × *g*, 45 min, 2°C). The supernatant, which contained F_1, was saved, and the pellet was resuspended in 0.27 ml of the same buffer. After 3 hr on ice, centrifugation was repeated, and the supernatants were combined. One-third of the pellet was resuspended in 0.22 ml of 10 mM Hepes, pH 7.5/50 mM NaCl/10 mM $MgCl_2$/1 mM dithiothreitol (IMV buffer). Another one-third of the pellet was resuspended in one-third of the combined supernatants (0.21 ml), then brought to 10 mM Hepes, pH 7.5/50 mM NaCl/10 mM $MgCl_2$/1 mM dithiothreitol, by the addition of 2.2 μl of each of the following: 1 M Hepes (pH 7.5), 5 M NaCl, 1 M $MgCl_2$, and 0.1 M dithiothreitol. The vesicles were incubated 15 hr on ice, centrifuged as described above, and resuspended to 11 mg/ml in IMV buffer.

Other Methods. Membrane concentrations are expressed as protein concentrations and were measured as described (19). Fluorograms were quantified by alkali elution of silver grains according to Suissa (20). All values are expressed as mean ± 1 SD.

RESULTS

^{35}S-labeled pro-OmpA was synthesized in a cell-free reaction mixture and filtered through Sephadex G-25 (see schematic representation in Fig. 1). The pro-OmpA was then mixed with isolated inverted plasma membrane vesicles. The active site of bacterial leader peptidase faces the periplasmic surface of the plasma membrane (8, 21), which corresponds to the lumenal surface in these vesicles (10). An electrochemical potential can be generated across these membranes by at least two enzymes, the F_1F_0-ATPase and NADH oxidase. Under appropriate assay conditions, pro-OmpA was translocated into the vesicles and processed to OmpA. In most experiments, vesicles were then incubated with protease to assay for translocation. OmpA and pro-OmpA were analyzed by immunoprecipitation, $NaDodSO_4$/PAGE, and fluorography.

When pro-OmpA (Fig. 2, lane 7) was incubated with membrane vesicles without an energy source, 44% was processed to OmpA (lane 1). However, <5% of this OmpA was protected from digestion by chymotrypsin (lane 2). This reaction has been observed before (10); it may be due to unsealed membranes or to a partial translocation reaction. When nucleoside triphosphates were added to the incubation, the same proportion of the pro-OmpA was cleaved to OmpA (lane 4) but 33% of the pro-OmpA, as well as 58% of the OmpA, was protected from digestion by chymotrypsin

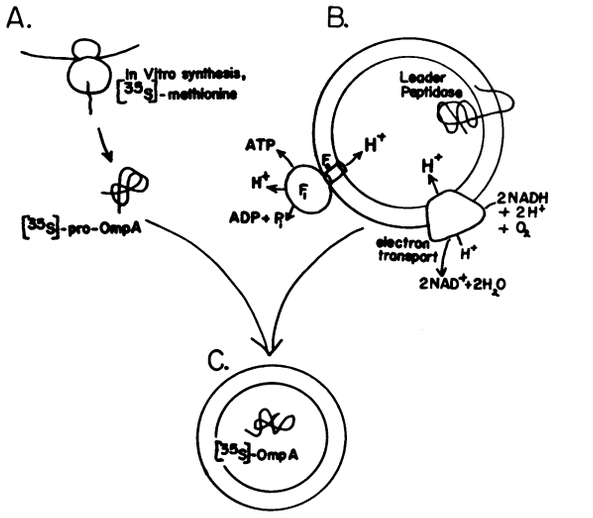

FIG. 1. Measurement of the translocation of pro-OmpA and sequestration of OmpA. (*A*) ^{35}S-labeled pro-OmpA was synthesized in a cell-free reaction and filtered through Sephadex G-25. (*B*) Inverted inner membrane vesicles contain leader peptidase, ATPase, and NADH oxidase. The active site of leader peptidase is inside the vesicles. ATPase and NADH oxidase energize the vesicles by creating an electrochemical potential. (*C*) ^{35}S-labeled pro-OmpA and the inverted inner membrane vesicles were mixed. Pro-OmpA was translocated and converted to OmpA. Chymotrypsin was added to assay the OmpA, which has translocated through the vesicle membrane.

(lane 5). Nearly complete digestion was observed when the membranes were lysed by detergent (lane 6). Translocation into sealed vesicles is thus completely dependent on the addition of an energy supply. In all subsequent experiments (Figs. 3–5), samples were therefore digested with protease prior to immunoprecipitation; thus only the protected OmpA and pro-OmpA were assayed.

With inner membrane vesicles from wild-type cells, ATP was sufficient for maximal translocation, assayed as protease-inaccessible OmpA (Fig. 3, lane 2). NADH did not support translocation (lane 4) and did not stimulate the

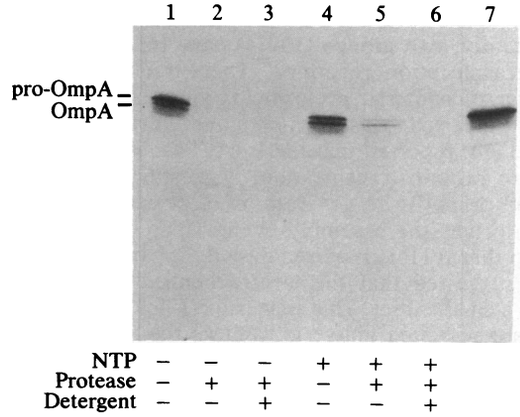

FIG. 2. Translocation and sequestration of OmpA requires energy. ^{35}S-labeled pro-OmpA (lane 7) was mixed with inverted inner membrane vesicles (90 μg/ml) and incubated without energy source (lanes 1–3) or with 1 mM ATP/GTP/CTP/UTP plus pyruvate kinase (4 μg/ml) and phospho*enol*pyruvate (4 mg/ml) (lanes 4–7). The mixtures were cooled to 0°C, and chymotrypsin and octyl-β-D-glucopyranoside were added as indicated. After 1 hr at 0°C, chymotrypsin inhibitors were added. Aliquots were immunoprecipitated with anti-OmpA antiserum and analyzed by $NaDodSO_4$/PAGE and fluorography.

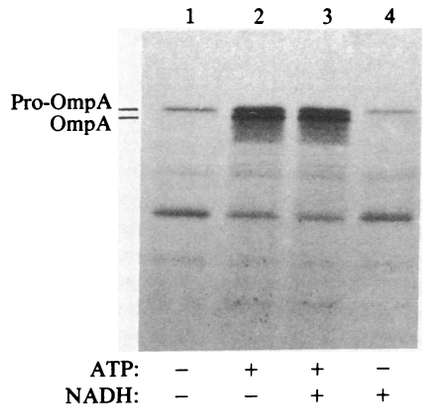

FIG. 3. Membrane potential alone is not sufficient for translocation. ^{35}S-labeled pro-OmpA was incubated with membrane vesicles (300 μg/ml). The mixtures contained ATP and/or NADH as indicated. After incubation, the samples were treated with protease as described and analyzed by $NaDodSO_4$/PAGE and fluorography.

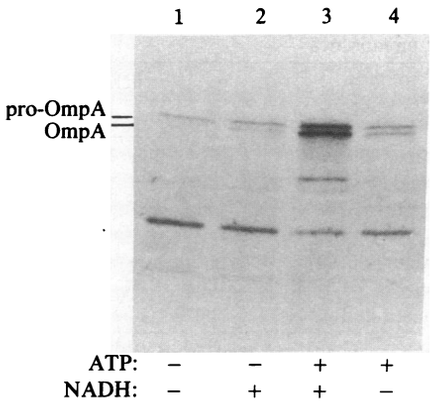

FIG. 4. ATPase deleted membranes require both ATP and NADH for optimal translocation. ^{35}S-labeled pro-OmpA was mixed and incubated with membrane vesicle (300 μg/ml) made from DK8, a strain that lacks the F_1F_0-ATPase. The mixtures contained ATP and/or NADH as indicated. After incubation, the samples were treated with protease as described and analyzed by $NaDodSO_4$/PAGE and fluorography.

translocation seen with ATP alone (lane 3). Since the data in Fig. 3 (and also in Figs. 4 and 5) show immunoprecipitates that were done after protease digestion, only the full-length pro-OmpA and mature-length OmpA that were inaccessible to protease are seen. The other bands below OmpA are proteolytic fragments generated from the digestion of protease-accessible pro-OmpA and OmpA.

In these membranes, either NADH oxidation or ATP hydrolysis will generate a membrane electrochemical potential, assayed (Table 1, rows 1–3) as the quenching of 9-amino-6-chloro-2-methoxyacridine fluorescence (18). Cyanide, an inhibitor of the electron transport chain, blocked the NADH-induced potential, while the uncoupler CCCP dissipated the potential induced by ATP. Either NADH or ATP can generate a comparable potential, yet ATP alone can support translocation, while NADH cannot. These results support the conclusion of Chen and Tai (11) that the membrane potential alone is not sufficient for protein translocation. These results do not indicate whether the potential plays a necessary role.

To separate the direct effects of ATP on translocation from its role in generating a membrane potential, the F_1F_0-ATPase was eliminated from the reaction. This was achieved by using membrane vesicles from DK8, a strain in which the *atp* operon has been entirely deleted. As expected, inverted plasma membrane vesicles from DK8 can use NADH, but not ATP, to generate a membrane potential (Table 1). In the presence of NADH and ATP, these vesicles translocate and process pro-OmpA to yield sequestered OmpA (Fig. 4, lane 3). Either ATP or NADH alone (lanes 2 and 4) only supports 20% of the assembly seen when both are present, while no assembly is seen in the absence of an added energy source (lane 1). These data clearly indicate that both the membrane potential and ATP are necessary for optimal pro-OmpA assembly into these vesicles.

To confirm this result, F_1-ATPase was "stripped" from the membranes by incubation in a buffer of low ionic strength with EDTA. The F_0-ATPase remains with the membranes and acts as a proton-specific channel. These membranes were therefore incapable of maintaining a potential generated through either oxidation of NADH or hydrolysis of ATP (Table 2). Even in the presence of both ATP and NADH, they processed and sequestered very little pro-OmpA (Fig. 5, lane 3). A portion of the stripped membranes was reconstituted by adding back the stripped proteins, thereby sealing the F_0 channels and restoring the capacity to generate and maintain a potential in response to ATP or NADH (Table 2). Reconstituted membranes regained pro-OmpA processing and protection (Fig. 5, lanes 5 and 6).

It was possible that the stripping procedure might have removed or inactivated a membrane component (other than F_1) that was needed for pro-OmpA translocation and processing. To test this, we stripped membrane vesicles from DK8 (the ATPase deletion strain). There was no effect on the ability of these membranes to generate a membrane potential or process and sequester OmpA (data not shown). Therefore, it is the stripping and reconstitution of the F_1-ATPase from D10 membranes that fully accounts for the effects shown in Fig. 5. These results directly demonstrate that ATP alone will not support pro-OmpA translocation; the membrane potential is also required.

Table 1. NADH- and ATP-dependent membrane energization

Strain	Membranes, μg	Electrochemical potential				
		No energy	+ NADH	+ NaCN	+ ATP	+ CCCP
1. No membranes	0	100	92	95	99	109
2. D10 (wild-type)	22	100	50	117	80	127
3. D10	44	100	35	97	38	102
4. DK8 (ATPase$^-$)	10	100	46	98	92	103
5. DK8	40	100	3	113	113	119

The membrane electrochemical potential (relative fluorescence) was measured by fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine as described (18), except the assay buffer was 50 mM sodium morpholinopropane sulfonate/10 mM $MgCl_2$, pH 7.3. All measurements were made using an Aminco SPF 500 fluorometer. Initial fluorescence of the membranes in buffer was set at 0%. 9-Amino-6-chloro-2-methoxyacridine (1 μM) was added, and this level of fluorescence was set to 100%. Fluorescence quenching was measured after the sequential addition of each of the following: 0.5 mM NADH, 1.5 mM NaCN, 0.4 mM ATP, and 2.5 μM CCCP.

Table 2. NADH- and ATP-dependent energization of F_1-stripped membranes

	Electrochemical potential		
	No energy	+ NADH	+ NaCN
A. Membranes (20 μg)			
Stripped	100	97	92
Reconstituted	100	85	94
B. Membranes (40 μg)	No energy	+ ATP	+ CCCP
Stripped	100	98	104
Reconstituted	100	64	102

The F_1-ATPase was stripped from the membrane vesicles as described. Half of the membranes were reconstituted to NADH- and ATP-dependent energization competency by adding back the stripped F_1-ATPase. The electrochemical potential (relative fluorescence) was measured as described in Table 1. In part B, no NADH or NaCN was added prior to ATP and CCCP.

DISCUSSION

Our previous *in vivo* studies (5, 22) have shown that the membrane assembly of M13 procoat protein is sensitive to CCCP and other uncouplers. In these studies, it was demonstrated that the membrane potential is not simply needed to drive the synthesis of ATP. Periplasmic and outer membrane proteins also directly require the membrane potential for their export (6, 7, 9). The current studies indicate that there are, at least *in vitro*, two separable energy requirements for bacterial protein translocation: ATP and a membrane electrochemical potential. It will be of interest to determine whether protein uptake into mitochondria, which is potential dependent, and into chloroplasts, which is ATP dependent, may also in fact require both ATP and the membrane potential.

In this report, we confirmed the finding of Müller and Blobel (14), that the stripping of the F_1 renders the membranes nearly incapable of both generating a membrane potential (Table 2) and translocating and sequestering OmpA (Fig. 5). In addition, we also showed that the membrane potential alone is not sufficient for optimal translocation (Fig. 3, lane 4; Fig. 5, lane 4). Our results with the ATPase deletion strain differ only quantitatively from those of Chen and Tai (11). We found that ATP alone supports OmpA translocation poorly (Fig. 4, lane 4), whereas Chen and Tai found ATP to be nearly optimal (table 2 in ref. 11). The discrepancy may be due to the differences in the strains. They used ATPase mutant strains having a point mutation in one of the ATPase subunits, which may only partially inactivate the ability of the membrane vesicles to use ATP to generate a membrane potential. We have used a strain, DK8, that lacks the entire *atp* operon, and we have shown (Table 1, lines 4 and 5) that ATP does not generate a membrane potential in these vesicles. We conclude that neither ATP nor a membrane potential alone is sufficient and that both are necessary for optimal translocation and sequestration of OmpA.

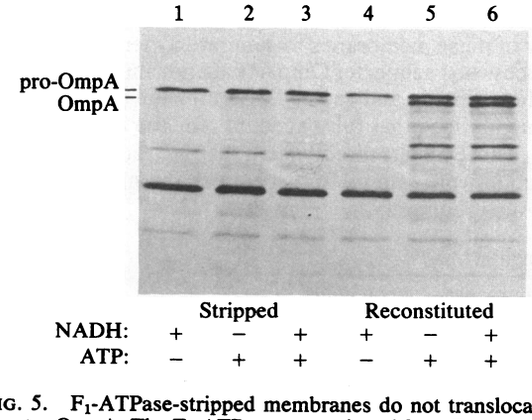

FIG. 5. F_1-ATPase-stripped membranes do not translocate and sequester OmpA. The F_1-ATPase was stripped from D10 membrane vesicles (18). Half of the stripped vesicles were reconstituted by incubating with the F_1-ATPase that had been removed. ^{35}S-labeled pro-OmpA was incubated with stripped (lanes 1–3) and reconstituted (lanes 4–6) vesicles (240 μg/ml). The mixtures contained ATP and/or NADH as indicated. After incubation, the mixtures were treated with protease, immunoprecipitated, and analyzed by $NaDodSO_4$/PAGE and fluorography as described.

We do not know what role either the potential or ATP plays in protein translocation through the bacterial plasma membrane. Nonhydrolyzable ATP analogs not only fail to support translocation (11) but act as competitive inhibitors of pro-OmpA translocation (B.L.G., unpublished data), suggesting that ATP hydrolysis is linked to protein export. The observation that vesicles that entirely lack the F_1F_0-ATPase can support protein translocation should simplify efforts to track the fate of the γ-phosphoryl group from ATP in this reaction.

We thank Dr. P. C. Tai for stimulating discussions and Dr. R. D. Simoni for bacterial strains and guidance in fluorescence assays. Expert technical assistance was provided by Marilyn Rice and Douglas Geissert. B.L.G. is a fellow of the Atherosclerosis Training Program (U.S. Public Health Service HL 07386). This work was supported by a grant from the National Institutes of Health.

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